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Lethal dose, 50%

OBSERVATIONS ON A VENOM NEUTRALIZING ALBUMIN ENRICHED FRACTION ISOLATED FROM SERUM OF THE NORTHERN COPPERHEAD AGKISTRODON CONTORTRIX MOKASEN

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Venom neutralizing capacity of serum from the northern copperhead Agkistrodon contortrix mokasen was studied. Crude serum neutralized the hemorrhagic, proteolytic and lethal activity of A. c. mokasen venom. The serum neutralized up to 2.5 LD_{50} of A. c. mokasen venom and up to 5 LD_{50} of A. piscivorus venom. The serum was anti-hemorrhagic and a potent inhibitor of venom proteases and trypsin. An albumin enriched fraction was isolated from A. c. mokasen serum by DEAE- Affi - Gel Blue chromatography and Superose-12 (molecular sieve) fast protein liquid chromatography with recycling. This fraction contained two major proteins with apparent molecular weights of 62 kD and 79 kD respectively and several contaminants with an M_r from 52 - 79 kD. The fraction had a pI of 4.6-4.7 and displayed anodal migration in horizontal electrophoresis. The albumin enriched fraction exhibited the venom neutralization capacity of the crude serum, but did not inhibit venom proteases or trypsin. In amounts of only 16.5 µg, it protected against hemorrhage induced by up to 100 µg of A. c. mokasen venom. The venom neutralizing activity of serum from Lampropeltis getulus getulus is also discussed.

INTRODUCTION

The resistance of venomous snakes to their own venoms has been an avenue of investigation since the late eighteenth century. As early as 1787, Fontana observed that vipers were immune to their own venom and to venom from other members of the same species. Kellaway (1937) found marked autoimmunity in Notechis and described resistance of Notechis to venom of Austrelaps. Gloyd (1933) and Swanson (1946) independently reported that Agkistrodon piscivorus lacked immunity to it's own venom. Clark and Voris (1969) found that an enriched albumin fraction of Crotalus adamanteus serum was protective in mice when injected prior to administration of a lethal dose of C. adamanteus venom. Omori-Satoh et al. (1972) described an anti-hemorrhagic fraction of Trimeresurus flavoviridis serum which migrated immunoelectrophoretically as albumin or α_1-globulin. Straight et al. (1976) associated venom neutralizing activity of C. adamanteus serum with a serum protein other than albumin. Philpot et al. (1978) considered the neutralizing capacity of Agkistrodon and Crotalus sera to be associated with an inhibitor of venom proteases. Ovadia (1978) identified an anti-hemorrhagic fraction of Vipera palaestinae serum as an α-globulin or albumin-like component. This fraction also neutralized the hemorrhagic activities of Cerastes cerastes and Echis coloratus venoms. Nahas et al. (1983) found that plasma of Bothrops jararaca and Waglerophis merriami were resistant to clotting induced by a number of different ophidian venoms. These plasmas also inactivated clotting activity of these venoms upon human plasma. Recently, Tomihara et al. (1988) characterized a hemorrhagin neutralizing factor from serum of the colubrid, Dinodon semicarinatus. The present study describes characteristics of a venom neutralizing albumin enriched fraction isolated from serum of the northern copperhead Agkistrodon contortrix mokasen.

MATERIALS AND METHODS

Serum and Venom Samples

Adult specimens of the northern copperhead Agkistrodon c. mokasen were collected in Sullivan Co., N.Y. and Frederick Co., MD. Specimens of the southern copperhead A. c. contortrix were of unknown provenance. Specimens of the common eastern king snake, Lampropeltis getulus getulus, were commercially obtained long-term captives. All specimens were maintained on a diet of freshly killed Swiss-Webster mice and were fasted for 10-14 days prior to removal of blood samples. Each snake was anesthetized with ketamine hydrochloride (50 mg/kg) injected intramuscularly. Cardiac puncture was performed with a 26 1/2 gauge needle in order to obtain an average of 0.8 ml blood per snake. Blood was allowed to clot for 3-6 hr at 4^{0}C then centrifuged at 14,000 rpm for 5 min. The serum was siphoned off and any sample not used immediately was frozen at - 20^{0}C.

Additional samples of A. c. mokasen serum (analyzed separately) were kindly supplied by Dr. Sherman A. Minton. Venom was extracted as a pool from specimens of A. c. mokasen collected in New York, Indiana and Maryland. Eastern cottonmouth (A. piscivorus) venom was collected from a single long term captive originally from Tampa, Florida. Venom was collected every 3 weeks, immediately frozen, lyophilized, and stored over desiccant at 4^{0}C in the dark. Western diamondback rattlesnake (Crotalus atrox) venom was collected as a pool from a large number (>100) of specimens gathered in Archer County, Texas. The venom was lyophilized and stored as described above.

DEAE affinity chromatography

DEAE anion exchange affinity chromatography was performed in an Econo-column (Bio-Rad Laboratories Richmond, CA.) 0.5 x 15 cm packed with DEAE Affi-Gel Blue (Bio-Rad Labs.) Serum samples (typically 1.0-1.2 ml) were dialyzed against Tris-HCl 0.02 M, pH 8.0 containing 0.15 M NaCl and 0.02 % NaN_3 (column buffer) at 4^{0}C for 24 hr. The dialyzed serum was filtered through a 0.22 µ millipore membrane and applied to the column. Chromatography was carried out at 4^{0}C with a flow rate of 32 ml/hr. After 3 bed volumes of buffer had passed through the column, elution was initiated by establishing a linear gradient with column buffer containing 1.2 M NaCl. Total gradient volume was 150 ml. Eluent was monitored at 280 nm and fractions of 2.5 ml were

collected. Fractions exhibiting anti - proteolytic and/or anti - hemorrhagic activity were pooled for furthur analysis.

Fast Protein Liquid Chromatography (FPLC)

Anti - hemorrhagic and anti - proteolytic fractions obtained from affinity chromatography were concentrated using an Amicon ultrafiltration cell (Amicon Laboratories, Lexington, MA) with a 10 kD exclusion limit membrane. The fraction pool was then injected in 600 µl aliquots onto an FPLC System (Pharmacia Laboratories, Piscataway, NJ) equipped with a Superose-12 H_r 10/30 (molecular sieve) column. The column was developed with Tris-HCl 0.05 M, pH 7.2 containing 0.7 M NaCl. The column was run with an established flow rate of 0.5 ml/min and fractions of 1.0 ml were collected. The eluate was monitored at 280 nm and corresponding fractions of high absorbance were pooled.

Lethal potency determination

The intraperitoneal (i.p.) LD_{50} of crude A. c. mokasen venom was obtained by injection of male Swiss-Webster mice (18-20 gm) in groups of 4 mice per group. All injections were administered in the lower quadrants of the abdomen. Dosage was derived from a 1 mg/ml solution of venom in phosphate buffered saline (PBS), pH 7.2. Animals were observed after injection and mortality recorded after 24 hr. Animals succumbing to venom dosages were necropsied and any gross tissue pathology examined. The LD_{50} was calculated by the Spearman-Karber method (World Health Organization, 1981). The fiducial limits for the LD_{50} were determined. The i.p. LD_{50} of crude A. piscivorus venom was determined from a published value (Minton, 1974). The value was confirmed at 4 dosage levels. The LD_{50} of crude Crotalus atrox venom was determined as described above.

Protein determination

Protein content was estimated by using the bicinchoninic acid assay (BCA assay, Pierce Chemicals, Rockford, IL) (Smith et al., 1985).

Proteolytic activity

Proteolytic activity was assayed using a Bio-Rad Protease substrate Kit (Bio-Rad Laboratories, Richmond, CA). Tablets consisting of casein and 1% agarose in phosphate buffered saline (PBS), pH 7.2 were melted and poured onto a plastic plate

(85x65x2 mm). Wells were punched into the plate and dilutions of venom and/or serum or albumin enriched fractions ranging from 1-60 µg were applied (7-10 µl/well). Trypsin (5µg) served as a positive control and standard. Venom, serum and trypsin samples were diluted with PBS, and a well filled with PBS served as a negative control. The assay was run for 20 hr at room temperature. The reaction was stopped by addition of 3% acetic acid and areas of clearing were measured.

Ouchterlony double immunodiffusion

Ouchterlony assays were carried out in 1% agarose in PBS (pH 7.2). Serum or albumin enriched samples were applied in wells punched in the gel. Each well was filled with 10 µl (10 µg) of each sample and a center well was filled twice with 10 µl of anti-human serum albumin antiserum, anti-bovine serum albumin antiserum, or venom samples (20 µg total). The gel was covered and allowed to develop for 24 hr at room temperature, or 48 hr at 4°C. After incubation the plates were washed with PBS (four changes, 100 ml apiece), stained with amidoblack or Coomassie Blue R-250, then examined under light microscopy.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a Protean II mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA) using a 12.5% gel according to the method of Laemmli (1970). Electrophoresis was carried out at 100V, 25 mA for 55-65 min. The gel was then fixed in methanol-acetic acid and stained with either coomassie blue R-250 or silver nitrate. Mr was calculated by Andrews plot.

Isoelectric focusing (IEF)

IEF was performed in a 110 ml electrofocusing column (Pharmacia - LKB Instruments, Piscataway, NJ). The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 8% (w/v) ampholine (pH 3-10) and a more dense solution consisting of dialyzed sample, 8.5 ml of 8% (w/v) ampholine (pH 3-10) and 25 gm of sucrose in a final volume of 55 ml. Focusing was carried out at 4°C for 24 hr with a final potential of 1000 V. Eluate was monitored at 280 nm and fractions of 4 ml were collected.

Hemorrhagic assay

Hemorrhagic effect of titrated venom dilutions and the effect of serum dilutions upon venom induced hemorrhage were determined following the protocol of Kondo et al. (1960). The assay was performed in anesthetized (pentobarbital sodium, 25 mg/kg) Sprague-Dawley rats (200-250 gm) with venom dosages 25 mg-100 mg injected intradermally. Serum samples (62 µg - 250 µg) or albumin enriched fractions (3 µg - 20) were injected either alone or with appropriate venom dilutions. Serum or serum fractions mixed with venom samples were incubated at 37°C for 45 min to 1 hr prior to injection. The assay was allowed to proceed for 18 hr.

Venom neutralization assays

Venom neutralizing capacity of A. c. mokasen serum was assayed by mixing dilutions of serum ranging from 1.2-1.3 mg serum protein with dilutions of venom ranging from 120-475 µg (maximum mixed volume 300 ml), incubating at 37°C for 1 hr, then injecting the mixture (i.p.) into male Swiss-Webster mice (18-20 gm). Hamilton syringes (500 µl) were used for all injections. Some animals were injected with appropriate dilutions of serum prior to venom administration. Controls consisted of animals injected with volumes of bovine serum albumin (BSA) mixed with PBS and BSA mixed with appropriate venom dilutions. Volumes of control injections were equal to those of venom and mokasen serum or albumin enriched fractions. Animals were observed and mortality recorded after 24 hr. Neutralization capacity of mokasen serum fractions was investigated by mixing dilutions of albumin enriched fractions ranging from 1 µg - 1 mg with venom dilutions ranging from 120-450 µg (maximum mixed volume was 300 µl), incubating at 37°C for 1 hr, then injecting the mixture as described for the crude serum. Controls were performed as described above. Animals succumbing to venom injections were necropsied and gross tissue pathology examined.

RESULTS

The i.p. LD_{50} of A. c. mokasen venom was 6.5 mg/kg, and a published (Minton, 1974) LD_{50} value (5.11 mg/kg) for A. piscivorus venom was checked for possible variation and found to correspond closely with venom obtained from a single A. piscivorus specimen. Mice injected with lethal doses of A. c. mokasen venom showed prostration, lethargy and marked hemorrhage. Necropsy of animals succumbing to

doses of crude venom displayed diffuse hemorrhage at the injection site, mesenteric hemorrhage and extensive extravasation. Animals succumbing to doses of A. piscivorus venom appeared similar but upon necropsy showed a greater volume of sero-sanguinous fluid. Injection of 1.3 mg A. c. mokasen serum protein with 2.5 LD_{50} (327.5 µg) of A. c. mokasen venom resulted in 100% survival (Table I). Mice injected with the mixture exhibited some of the symptomology observed in animals injected with a single LD_{50} dose of venom alone, however these symptoms were of much shorter duration and resulted in almost complete recovery (some animals showed persistent inflammation which hindered full recovery). Several animals were sacrificed and examined, and were found to have a small zone of hemorrhage limited to the site of injection. Administration of up to 5 LD_{50} of A. piscivorus venom with 1.3 mg A. c. mokasen serum protein resulted in 100% survival (Table I). Wyeth polyvalent anti-crotaline antiserum was examined for comparative neutralization capacity. Injection of 1 mg of antivenin with 2.0 LD_{50} of A. c. mokasen venom resulted in 100% mortality while injection of 1.5 LD_{50} resulted in 100% survival (Table I). In contrast, 1 mg of antivenin was found to neutralize up to 2.0 LD_{50} of A. piscivorus venom (Table I). Administration of 2.1 mg of Lampropeltis getulus getulus serum protein protected against 1.5 LD_{50} of A. c. mokasen venom and 5 LD_{50} of A. piscivorus venom (Table I). In addition, no protection was observed when 1 3 mg of A. c. mokasen serum was administered with 1.5 LD_{50} of Crotalus atrox venom (Table I).

The inhibitory effect of crude A. c. mokasen serum upon venom proteolysis is summarized in Table II. A. c. mokasen crude serum (29 µg protein) incubated with up to 10 µg A. c. mokasen venom resulted in 100% inhibition of caseinolytic activity. When venom concentrations were increased to 20 µg some proteolysis occurred. However, this was only 10% of the proteolysis observed with 20 µg of venom alone. Table II also shows that A. piscivorus venom caseinolytic activity was identically inhibited by A. c. mokasen serum. The inhibitory effect upon venom proteases by Lampropeltis getulus getulus serum (60 µg) was also noted, as was the inability of commercial antivenin to inhibit venom proteolysis. It is noteworthy that trypsin (5-10 µg) was inhibited 100% by A. c. mokasen serum.

Table III shows the effect of crude A. c. mokasen serum upon A. c. mokasen venom induced hemorrhage. The hemorrhagic effect of 25 µg of venom with serum was significantly inhibited, while 50 µg of venom with serum was disproportionately inhibited. The effect of 100 µg of venom with serum was still inhibited by over 50%.

Application of 1.25 ml of dialyzed A. c. mokasen serum to a DEAE - Affi - Gel Blue column resulted in a small γ-globulin peak (peak 1) present in the void volume and one large peak (peak 2) with two prominent shoulders which eluted with 0.40-0.45 M NaCl (Fig. 1). SDS-PAGE indicated peak 2 contained two enriched bands with apparent molecular weights of 62 kD and 79 kD as well as several contaminants with an M_r range of 52 - 79 kD (Fig. 2). In order to purify further the enriched fraction, the DEAE pool of the major species (fractions 47-52) was concentrated and applied to an FPLC Superose-12 (molecular sieve) column. FPLC resolved the DEAE pool into 3 additional peaks (Fig. 3). Of these, fractions 13-14 corresponded to the major component. These fractions were pooled and recycled on Superose-12 FPLC.The resulting profile showed a single large peak with a prominent shoulder (Fig. 4). Fractions 13-14 represented the major fraction.This fraction contained components that displayed properties of albumin (an M_r from 63 - 70 kD (Fig. 2), anodal migration in electrophoresis and a pI of 4.6-4.7). Table IV shows the purification, activity, and recovery of the albumin enriched fraction isolated from A. c. mokasen serum. Unlike the crude serum, the enriched fraction did not exhibit detectable protease activity. The fraction did not react with antiserum prepared against human serum albumin or bovine serum albumin when run on Ouchterlony double-diffusion or immunoelectrophoresis. A. c. mokasen venom did not form visible precipitin lines when reacted with the isolated albumin fraction. Fig. 2 also shows the SDS-PAGE profiles of crude A. c. mokasen and Lampropeltis g. getulus sera, as well as the crude venoms of A. c. mokasen and A. piscivorus.

Injection of a mixture of A. c. mokasen albumin enriched fraction (50 µg) and A. c. mokasen or A. piscivorus venom indicated that the fraction protected against up to 2.5 LD_{50} of either venom. Protection against 2.0 LD_{50} of A. piscivorus venom was observed with 40 µg of albumin enriched fraction. Injection of 75 µg of fraction two hours prior to injection of 2.0 LD_{50} of mokasen venom resulted in 100% survival, suggesting blockage of target receptors as a possible mechanism of protection afforded by this fraction against venom lethality. Animals injected with enriched fraction prior to venom administration displayed symptomology of crude venom injection for a more protracted period than animals injected with preincubated mixtures of enriched fraction and venom. Injection of 2 LD_{50} of A.c.mokasen venom followed 30 min later by administration of 1.56 mg of homologous serum protein resulted in 100% mortality. Administration of up to 20 mg of bovine serum albumin with or prior to injection of

venom, resulted in 100% mortality.

The albumin enriched fraction did not inhibit venom proteolytic activity (Table II). The DEAE pool contained some inhibitory activity, however only relatively high concentrations (24 μg) of this material afforded about 58% inhibition of venom proteolytic activity. As little as 16.5 μg of albumin enriched fraction prevented venom induced hemorrhage (Table III). Injection of enriched fraction mixed with 25-50 μg of A. c. mokasen venom resulted in complete lack of hemorrhage, while 100 μg venom with the fraction resulted in a hemorrhagic zone approximately 35.5% of that observed with 100 μg of venom alone.

DISCUSSION

Clark and Voris (1969) described an albumin fraction from Crotalus adamanteus serum which protected mice from lethal doses of Crotalus adamanteus venom. Their enriched fraction contained proteins in the 70-150 kD range which electrophoretically migrated with the mobility of albumin. Omori-Satoh et al., (1972) reported an antihemorrhagic fraction of Trimerusurus flavoviridis serum which had an approximate molecular weight of 70 kD and immunoelectrophoretically migrated similar to albumin and α-1 globulin. Straight et al., (1976) considered neutralizing activity of fractioned C. adamanteus serum as a protein other than an albumin fraction. Ovadia (1978) demonstrated antihemorrhagic activity in a fraction from Vipera palaestinae serum. The fraction had a molecular weight of 80 kD, a pI of 4.7 and was identified as an α-globulin or albumin like species. This material was twenty fold more effective in neutralizing V. palaestinae venom hemorrhagic activity than commercial antivenin, an observation that is similar to the data obtained from the present study. Similarly, Philpot et al. (1978) described a fraction from crotaline sera which protected mice from up to five LD_{50} of homologous venom and, unlike the fraction that we have described, was a strong inhibitor of venom proteases. This material ranged in molecular weight from 50-100 kD.

The venom neutralizing fraction isolated from serum of A. c. mokasen in this study contained two major protein species with apparent molecular weights of 62 kD and 79 kD accompanied by several contaminants , a pI of 4.6-4.7, migrated as albumin in horizontal electrophoresis and neutralized up to 2.5 LD_{50} of A. c. mokasen venom. It did not inhibit venom protease activity. The proteolytic inhibitor of the crude serum is probably a minor component, possibly in the γ-globulin peak. The protease inhibitory

capacity of the DEAE pool was probably due to contamination from the γ globulin peak. The albumin enriched fraction inhibited hemorrhagic activity by approximately 64-100%. Reaction of venom with serum or the enriched fraction did not produce visible precipitation.

The mechanism of neutralization appears to be a specific inhibition of venom induced hemorrhage. Experimental A. c. mokasen venom injection (i.p. or s.c.) usually results in edema, polymorphonuclear leukocyte infiltration, and limited hemorrhage (Wingert et al., 1980). Several investigators have isolated thrombin-like enzymes from A. c. contortrix venom (Markland et al., 1986; Markland and Guan, 1986; Egen et al., 1987).The hemorrhagic, proteolytic, and thrombin-like venom components are clearly the factors most important in lethal activity of mokasen venom. Philpot et al. (1978) found that the protective component of several crotaline sera prevented bradykinin release, an activity these workers associated with venom proteases. They also postulated that these serum components inhibited fibrinogenolytic activity. This is in agreement with the observations of Nahas et al. (1983) who reported inactivation of venom induced clot formation by serum of Bothrops jararaca. Data obtained from the present study supports neutralization by the described albumin enriched fraction of effects of non-proteolytic, hemorrhagic venom components. The protective effect of administration of the fraction prior to injection of venom suggests a possible blockage of target receptors as a potential mechanism for ophidian serum fraction mediated protection against venom lethality.

Ophidian serum components capable of neutralizing and/or inhibiting lethal activities have also been reported from colubrids (e.g., Lampropeltis getulus floridiana, Bennett and Guttman, 1971; L. getulus, Philpot et al., 1978; Dinodon semicarinatus, Tomihara et al., 1988; Waglerophis merremi, Nahas et al., 1983). Bennett and Guttman (1971) associated a γ-globulin component from Lampropeltis g. floridiana serum with neutralization of Agkistrodon piscivorus venom proteases. These investigators advanced the thesis that serum antibodies were capable of combining with venom proteases leading to inactivation of proteolytic activity.

Ophiophagic snakes probably evolved serum components with venom neutralizing properties in response to adaptive stress exerted by potential prey items, namely venomous snakes. In this instance, these snakes are able to prey upon venomous species and therefore compete with relatively few predators. Venom neutralizing serum fractions observed in non-ophiophagic venomous snake species such as the crotalines studied in this investigation, clearly play some other role. The venom resistance

afforded by these serum fractions is not in response to envenomation incurred during male combat or seasonal mating since biting among crotaline species is not observed during these activities (Carpenter, 1977a, 1977b). It can be considered to serve as protection against autoenvenomation (e.g., accidental envenomation during feeding) however this is presumably a rare event.

Shared antigens common to both serum and venom have been reported from several crotalines (Minton, 1973), elapids (Lamb, 1902; Tu and Ganthavorn, 1968) and between colubrid serum and Duvernoy's secretion from several opisthoglyphic colubrids and venom from the xenodontine colubrid, Heterodon platyrhinus, when reacted with anti-Dendroaspis antivenin (Minton and Weinstein, 1986). Most of the cross-reacting antigens observed in immunoelectrophoretic preparations of serum and venom exhibit beta-migration (Minton, 1979). A Naja venom fraction with phospholipase A activity has been found to be shared among sera of the Australian elapid, Austrelaps superbus and the hydrophiine elapid, Hydrophis melanocephalus (Minton, 1979). Alper and Balavitch (1976) described cobra venom factor from Naja naja venom as an altered form of C3 from Naja serum. The significance of these shared antigens among ophidian venoms and sera remains unclear.

Natural resistance to ophidian venoms has been reported also in scincid lizards (Minton and Minton, 1980), opposums Didelphis virginiana (Werner and Vick, 1977), hispid cotton rats Sigmodon hispidus (Pichyangkul and Perez, 1980), California ground squirrels Spermophilus beecheyi (Poran et al., 1987), mongooses Herpestes ichneumon (Ovadia and Kochva, 1977), Herpestes edwardsii (Tomihara et al., 1987), and bovine animals (Bolaños et al., 1975). Tomihara et al. (1987) isolated anti-hemorrhagic fractions from serum of the mongoose Herpestes edwardsii. Similarly, anti-hemorrhagins have been isolated and characterized from sera of the wood rat Neotoma micropus (Garcia and Perez, 1984), cotton rat Sigmodon hispidus (Pichyangkul and Perez, 1980) and European hedgehog Erinaceus europaeus (DeWit and Weström, 1987). Previous investigators have demonstrated a role for mammalian albumin function greater than previously considered (Lafaye and Lapresle, 1988; Baker, 1989). For example, human albumin may participate in a mechanism which modulates protection from drug allergy (Lafaye and Lapresle, 1988).

A potent protease inhibitor is present in a number of ophidian sera, however it does not seem to play a role critical to protection from A. c. mokasen venom lethal activity. Philpot et al., (1978) reported antiprotease inhibitors from fractions obtained from several crotaline sera using Sephadex G-200 chromatography. A fraction obtained from C. adamanteus serum was reported to neutralize A. c. contortrix venom.

The potent protease inhibitor from crude A. c. mokasen serum observed in the present study is noteworthy since venom proteases appear to be trypsin-like in activity (including their pH optimum and substrate specificity), but are not affected by conventional trypsin inhibitors (Philpot and Deutsch, 1956).

The lack of protection observed with mokasen albumin enriched fraction mixed with Crotalus atrox venom is compatable with data obtained from studies suggesting a rhexic hemorrhagic mechanism for C. atrox venom hemorrhagin action. Rhexic hemorrhage is characterized by destruction of the vascular wall, while hemorrhage per diapedesis is characterized by modification of intracellular junctions allowing blood components to escape without alteration of endothelial cell morphology (Ownby, 1982). Ownby et al. (1974) described C. atrox venom induced hemorrhage as rhexic in nature. Bjornson and Tu (1978) isolated five hemorrhagic toxins from venom of C. atrox. All five were found to possess proteolytic activity. Ownby et al. (1978) demonstrated that three of these (HTa, HTb, HTc) acted directly upon the capillary wall, causing degeneration of endothelial cells leading to plasma membrane rupture and ultimately extravasation. Ohtani et al. (1988) described a capillary permeability increasing kininogenase from venom of Agkistrodon caliginosus suggestive of a diapedetic mechanism. In addition, Ohtani and Takahashi (1987) reported a proteolytic enzyme from A. caliginosus venom which increased capillary permeability, and Sadahiro and Omori-Satoh (1980) demonstrated a hemorrhagic protease from venom of Trimeresurus flavoviridis. In contrast, Ohsaka et al. (1974) have shown that venom of T. flavoviridis caused diapedetic hemorrhage. It is also noteworthy that Fabiano and Tu (1981) described viriditoxin from venom of Crotalus viridis viridis as an acidic toxin with both hemorrhagic and myotoxic action.

While the precise mechanism and function of venom neutralizing components of ophidian serum remain to be elucidated, the potential for practical application appears clear. Production of synthetic peptides containing sequences derived from purified serum components could result in preparation of products superior in venom neutralization capacity then commercially produced antivenom. Such a preparation may present far less risk of immune complex disease than equine source antiserum and may be far more efficacious in preventing hemorrhage induced extravasation.

We are currently investigating the mechanism of neutralization using structure-function studies of individual purified components of the albumin fraction presently described. These investigations may illuminate the evolution and function of ophidian serum components and their role in the life histories of the snakes which possess them.

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LEGENDS

Fig. 1. CHROMATOGRAPHIC PROFILE OF CRUDE SERUM OF Agkistrodon contortrix mokasen APPLIED TO AFFINITY DEAE (ANION EXCHANGE) COLUMN.
Dialyzed serum (1.25 ml) was filtered through a 0.22 μ membrane and applied to a column (0.5 cm x 15 cm) packed with DEAE Affi-Gel Blue agarose. Chromatography was performed with Tris-HCL 0.02 M, pH 8 0 containing 0.15 M NaCl and 0.02% NaN_3 (column buffer) at 4^0C. Flow rate was maintained at 32 ml/hr and elution was performed with a linear gradient of 1.2 M NaCl in column buffer (total gradient volume = 150 ml). Fractions of 2.5 ml were collected and monitored for absorbance at 280 nm. Fractions exhibiting anti-hemorrhagic and/or anti-proteolytic activity were pooled and concentrated using an Amicon YM-10 ultra-filtration cell.

Fig. 2. SDS-PAGE OF CRUDE SERUM, ALBUMIN ENRICHED FRACTION, AND CRUDE VENOMS.
Isolation of albumin enriched fraction from serum of Agkistrodon c. mokasen was monitored throughout purification procedures (beginning with crude serum) by SDS-PAGE. The electrophoretic patterns of other ophidian sera (e.g. - Lampropeltis getulus getulus) and crude venoms (A. c. mokasen and A. piscivorus) were comparatively studied. Gels (12.5%) were cast and run using a BioRad mini Protean II unit according to the method of Laemmli (1970). Apparent molecular weights were determined by Andrews plots. Proteins were detected by staining with 0.1% Coomassie brilliant blue R-250. Lane 1 contains crude venom of A. piscivorus; lane2 crude venom of A. c. mokasen; lane 3 mol. wt. markers: phosphorylase b = 94,000, bovine serum albumin = 67,000, ovalbumin = 43,000, carbonic anhydrase = 30,000, soybean trypsin inhibitor = 20,100, α-lactalbumin = 14,400; lane 4 -Superose 12 (molecular sieve) FPLC, cycle 2, of Superose 12 FPLC, Cycle 1; lane 5 - Superose 12 (molecular sieve) FPLC, cycle 1, of Affinity DEAE pool; lane 6 -Affi-Gel pool; lane 7 crude serum of A. c. mokasen; lane 8 -crude serum of Lampropeltis getulus getulus.

Fig. 3. FPLC MOLECULAR SIEVE CHROMATOGRAPHY (CYCLE 1) OF AFFINITY DEAE (ANION EXCHANGE) ALBUMIN ENRICHED POOL.
Molecular sieve chromatography was carried out using a Superose 12 HR 10/30 column attached to the FPLC unit. Concentrated samples were injected into the column in 6 μl aliquots and a flow rate of 0.5 ml/minute was maintained. The column

was eluted with Tris HCl 0.05 M, pH 7.2 containing 0.7 M NaCl. All fractions (1.0 ml) were monitored for absorbance at 280 nm and examined for venom neutralization properties.

Fig. 4. FPLC MOLECULAR SIEVE CHROMATOGRAPHY (CYCLE 2) OF SUPEROSE-12 CYCLE 1.

Molecular sieve chromatography was carried out using a Superose 12 HR 10/30 column attached to the FPLC unit. Concentrated samples were injected into the column in 600 µl aliquots and a flow rate of 0.5 ml/minute was maintained. The column was eluted with Tris HCl 0.05 M, pH 7.2 containing 0.7 M NaCl. All fractions (1.0 ml) were monitored for absorbance at 280 nm and examined for venom neutralization properties.

Table 1. Neutralization by A. c. mokasen Serum of Venom Lethality

VENOM	A. c. mokasen serum (1.3 mg)	L. getulus getulus serum (2.1 mg)	Wyeth Antivenin (1 mg)
A. c. mokasen 1.5 LD_{50}	+	+	+
A. c. mokasen 2 LD_{50}	+	-	-
A. c. mokasen 2.5 LD_{50}	+	ND	ND
A. piscivorus 1.5 LD_{50}	ND	ND	+
A. piscivorus 2 LD_{50}	+	+	+
A. piscivorus 5 LD_{50}	+	+	ND
Crotalus atrox 1.5 LD_{50}	-	-	ND
Crotalus atrox 2 LD_{50}	-	-	ND

- = dead
+ = survived
ND = not determined

Table II. Inhibition by A. c. mokasen Serum and Albumin Enriched Fractions of Venom and Trypsin Proteolytic Activities

% [1] Inhibition

SAMPLE	A. c. mokasen serum (29 µg)	L. getulus getulus serum (60 µg)	A. c. mokasen albumin enriched fraction			Wyeth anti-crotaline antivenin (33µg)
			1.3 µg	1.7 µg	10 µg	
A. c. mokasen venom, 10 µg	100 %	75 %	0 %	0 %	0 %	0 %
A. c. mokasen venom, 20 µg	90 %	60 %	ND	ND	0 %	ND
A. piscivorus venom, 10 µg	100 %	100 %	0%	0 %	ND	0 %
A. piscivorus venom, 20 µg	87 %	ND	ND	ND	0 %	ND
Trypsin 5 µg	100 %	ND	0 %	0 %	0 %	ND

[1] Clearing zone (mm) produced in casein-agarose by venom with serum or albumin enriched fraction or trypsin with serum or albumin enriched fraction / clearing zone (mm) produced by venom or trypsin alone x 100

ND = not determined

Table III. Effect Upon Agkistrodon c. mokasen Venom Induced Hemorrhage of A. c. mokasen Serum and Serum Enriched Fractions

SAMPLE	AREA OF HEMORRHAGIC ZONE (mm)	INHIBITION COMPARED TO VENOM ALONE (%)
A. c. mokasen venom, 25 µg	6	-
A. c. mokasen venom, 50 µg	9	-
A. c. mokasen venom, 100 µg	12	-
A. c. mokasen venom, 25 µg + 62 µg A. c. mokasen serum	3	50 %
A. c. mokasen venom, 50 µg + 62 µg A. c. mokasen serum	6	66 %
A. c. mokasen venom, 100 µg + 62 µg A. c. mokasen serum	6.5 - 7	56.27 %
A. c. mokasen venom, 25 µg + 16.5 µg albumin enriched fraction	0	100 %
A. c. mokasen venom, 50 µg + 16.5 µg albumin enriched fraction	0	100 %
A. c. mokasen venom, 100 µg + 16.5 µg albumin enriched fraction	7.5 - 8	64.5 %
A. c. mokasen serum (62µg)	0	-
PBS, 20 ul	0	-

[1] % inhibition of hemorrhage is taken as the hemorrhagic zone (mm) resulting from injection of venom or venom and serum + albumin enriched fraction / hemorrhage zone (mm) resulting from venom alone x 100.

Table IV. Purification, Recovery, and Activity of Agkistrodon contortrix mokasen Serum and Albumin Enriched Fraction

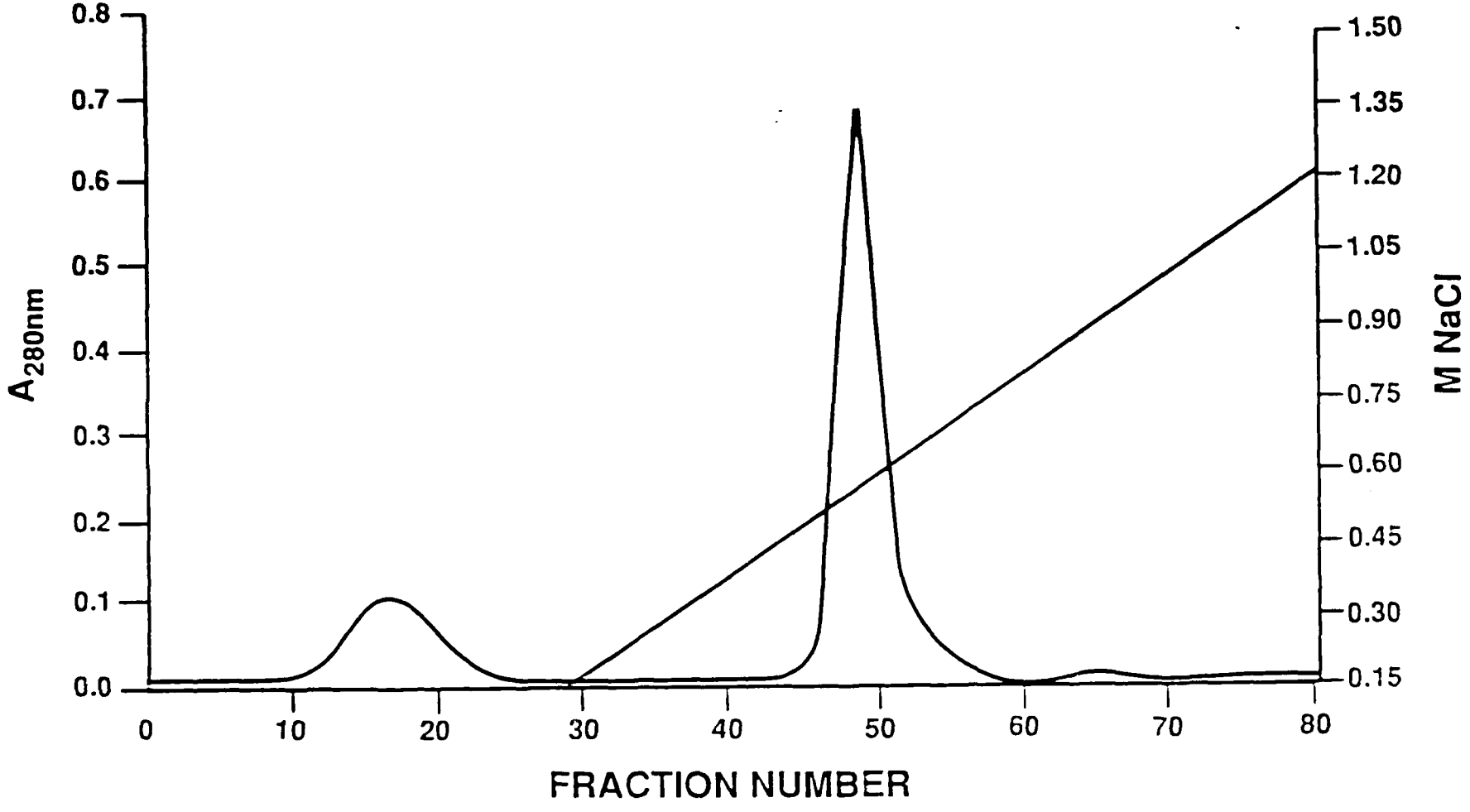
SAMPLE	Protein (mg / ml)	Volume (ml)	Total protein (mg)	Recovery (Protein)	[1] Specific Venom Neutralizing Activity (per mg)		[2] Inhibition of caseinolytic activity		[3] Inhibition of A. c. mokasen venom induced hemorrhage		
					A. c. mokasen venom	A. piscivorus venom	A. c. mokasen venom	A. piscivorus venom	25 µg	50 µg	100 µg
crude A. c. mokasen serum	52.3	1.25	65.3	100 %	2.2	4.4	100 %	100 %	90 %	65 %	40 %
DEAE-Affinity Pool (concentrated)	4.0	3.25	13.0	20 %	ND	ND	58 %	ND	ND	ND	ND
Superose 12 FPLC (cycle #1)	1.7	2.5	4.25	6.5 %	ND	ND	0	0	ND	ND	ND
Superose 12 FPLC (cycle #2)	0.36	2.7	0.972	1.5 %	40	50	0	0	100 %	100 %	64.5 %

[1] Neutralizing activity (= number of murine intraperitoneal venom LD_{50} neutralized / mg crude serum or albumin enriched fraction protein.

[2] Percentage inhibition of caseinolytic activity compared with area of clearing produced by venom alone (see Materials and Methods for details of the assay).

[3] Percentage inhibition of hemorrhagic zone produced by mixture of venom (25-100µg) and serum (62µg) or albumin enriched fraction (16.5µg) compared with zones produced by 25-100µg venom alone.

ND Not determined.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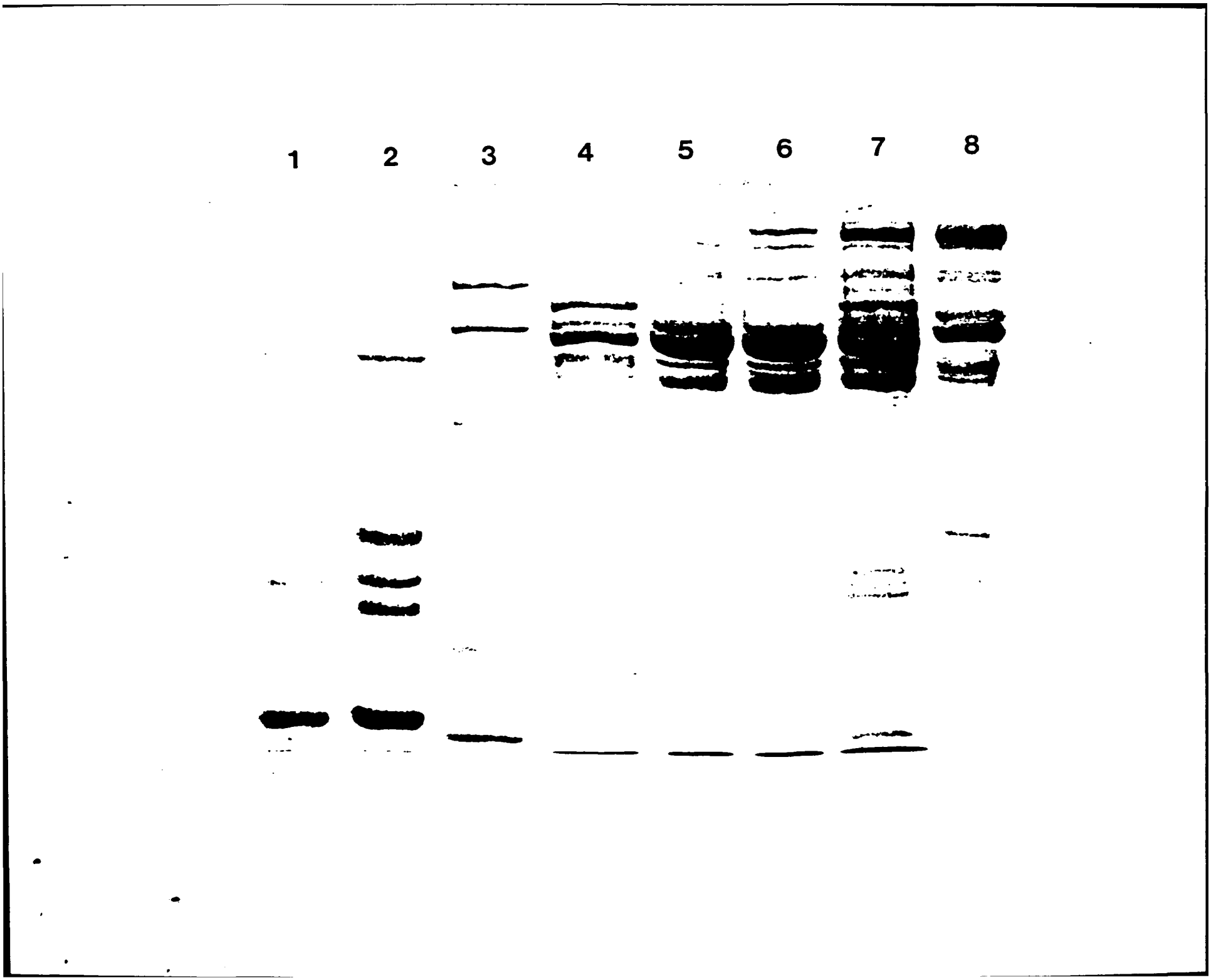
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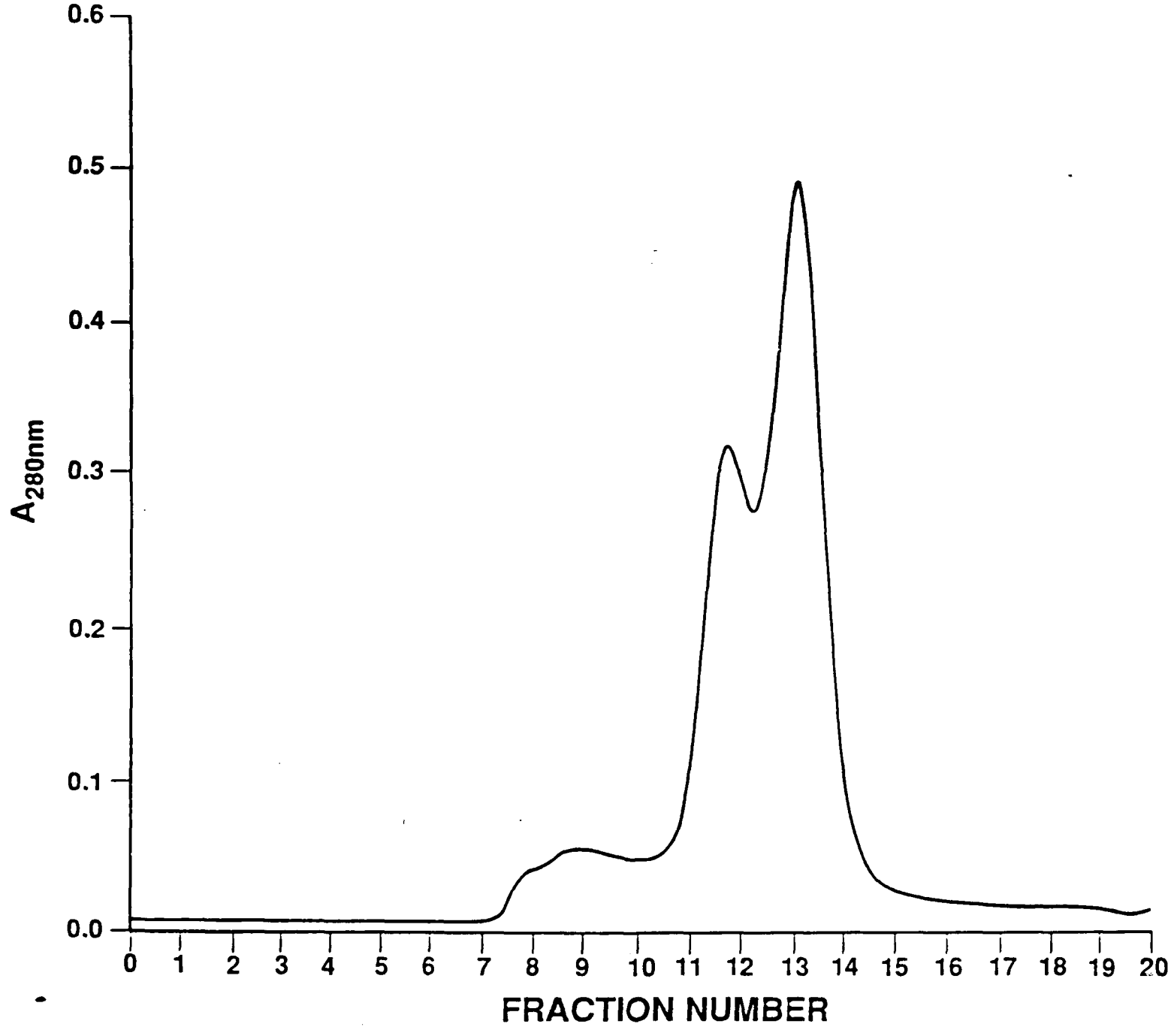
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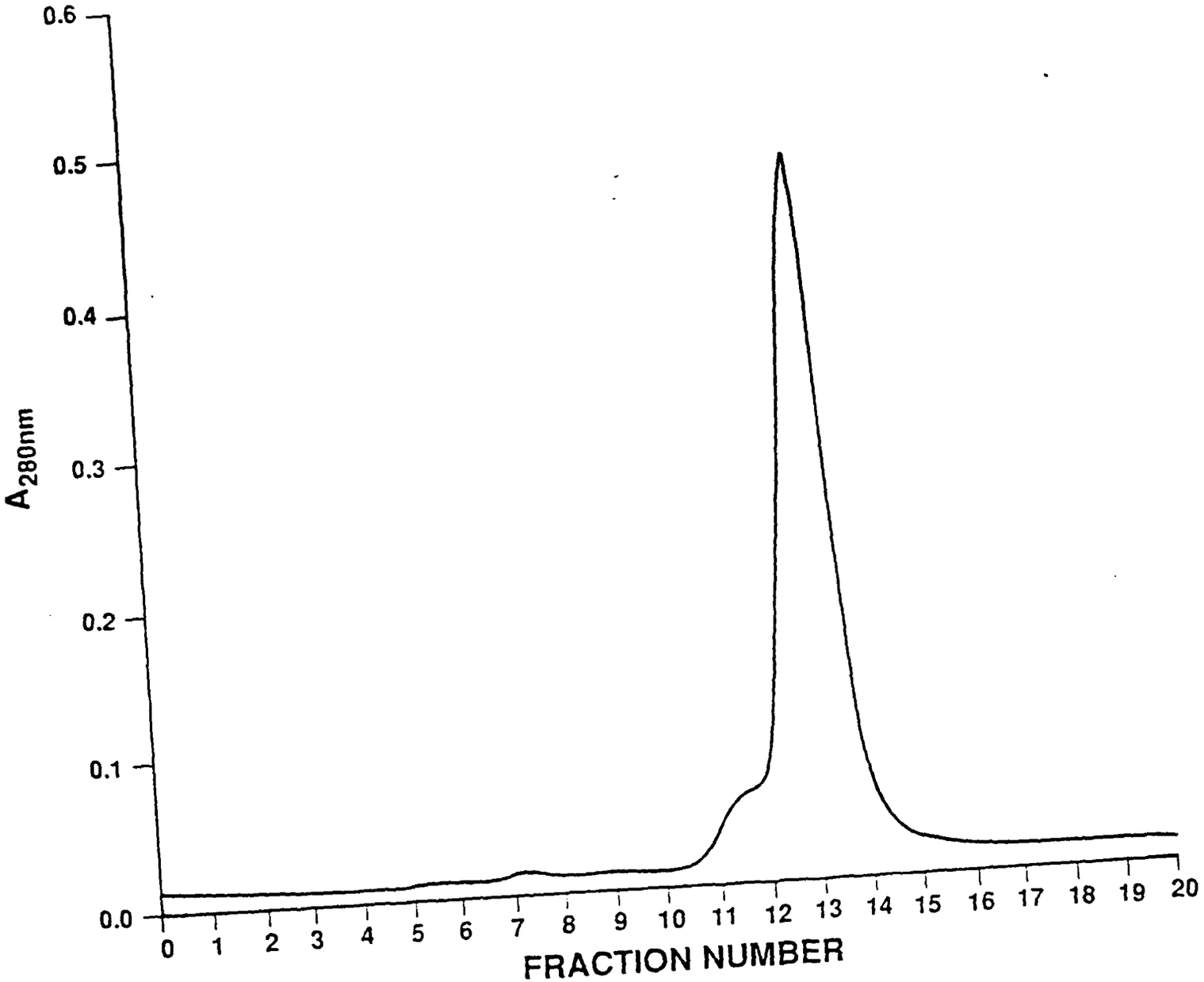
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